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Reduced Transcription and Progeny Virus Production of Hepatitis B Virus Containing an 8-bp Deletion in Basic Core Promoter

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We have demonstrated previously the presence of an 8-bp deletion mutant, spanning from nt. 1768 to nt. 1775 in the basic core promoter region of hepatitis B virus (HBV) in patients with anti-HBe positive asymptomatic phase before developing acute exacerbation after immunosuppressive treatment. The transcription and progeny virus production activities of the mutant were examined by transfection of the recombinant plasmid [pUC Del(2)] containing the head-to-tail dimer DNA of the mutant into HepG2 cells. The amounts of hepatitis B surface antigen (HBsAg) and HBe antigens secreted into the culture medium were markedly reduced. Southern blotting of DNAs extracted from the culture medium also showed reduced mutant activity to produce progeny virus. Northern blotting and RNase protection assay of RNAs extracted from transfected cells demonstrated that the transcription of both precore mRNA and pregenome RNA was reduced significantly compared to that of wild-type HBV. The promoter activity examined by transfection of the CAT plasmid containing deletion mutant DNA was much lower than that of wild type. Co-transfection experiments, however, of the CAT plasmid containing wild-type DNA with pUC Del(2) reduced CAT activity induced by wild-type, suggesting that truncated X protein produced by the mutant does not possess a sufficient transactivating activity. Gel shift assay using HepG2 nuclear extract and a probe containing four TA-rich regions in CP and various competitors suggested that the lack of the third TA-rich region was responsible for the transcription reduction of precore mRNA and pregenome RNA. The possible mechanisms are discussed. ***J. Med. Virol. 61:15–22, 2000.***

KEY WORDS: hepatitis B virus; core promoter; 8-bp deletion; transcription

INTRODUCTION

Hepatitis B virus (HBV), a member of the family *Hepadnaviridae* and a partially double-stranded DNA virus, causes acute and chronic infections. In natural infection of HBV, two major transcripts have been detected in infected hepatocytes; a subgenomic 2.1 kb mRNA encoding viral envelope antigen (HBsAg) and a 3.5 kb terminally redundant mRNA encoding nucleocapsid antigen (HBcAg) and e antigen (HBeAg). The polymerase with reverse transcriptase and RNase H activities is also coded by the latter mRNA. Thus, the 3.5 kbp mRNA plays an important role in viral replication and transcription. The 3.5 kbp mRNA consists of a long precore transcript (precore mRNA) and short pregenomic transcript (pregenome RNA). Both RNAs are transcribed under the control of a core promoter (CP) consisting of the basic core promoter (BCP) region and core upstream region (CUR), that also overlaps the enhancer II and several nuclear factor binding sites.

Recently, several genetic variants of HBV with mutations within the precore and/or core regions have been identified. Among HBV variants, a point mutation from G to A at nucleotide (nt.) 1896 (stop codon mutant) has been defined [Carman et al. 1989; Okamoto et al., 1990; Liang et al., 1991; Omata et al., 1991] and shown to exhibit enhanced viral replication activity resulting from increased stability of pregenome RNA [Lok et al., 1994; Yuan et al., 1995]. In addition, contiguous point mutations A to T at nt. 1762 and G to A at nt. 1764 (TA mutant) in the upstream region are predominant mutations found in a large number of patients with chronic hepatitis and in asymptomatic carriers after seroconversion [Laskus et al., 1994; Okamoto et al., 1994; Gotoh et al., 1995; Nishizono et al., 1995]. Although enhanced activity of TA mutant in progeny virus production has been demonstrated in previous studies, transcription levels of precore mRNA

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and pregenome RNA differ from one study to another [Buckwold et al., 1996; Moriyama et al., 1996; Günter et al., 1998].

The presence of another type of mutant HBV with deletion in CP has been demonstrated in several clinical phases of HBV infection [Laskus et al., 1994; Okamoto et al., 1994; Uchida et al., 1994; Fukuda et al., 1995, 1996; Günther et al., 1996; Nishizono et al., 1997]. The study of Moriyama [1997] was concerned with an 8-bp deletion mutant from nt. 1763 to nt. 1770 [Okamoto et al., 1994] and showed that the deletion is responsible for the reduced production of HBsAg, HBcAg and HBeAg, but it does not affect in vitro progeny virus production. Uchida et al. [1995] demonstrated that HBV mutant with 8-bp deletion from nt. 1770 to 1777 results in frameshift creating three novel amino acids followed by a new stop codon. Thus, x-gene protein was C-terminally truncated from 134 to 154 amino acid [Uchida et al., 1995]. We have also reported an 8-bp deletion mutant in CP from nt. 1768 to nt. 1775, with a deletion site different from the mutant described by Okamoto et al. [1994]. The mutant was derived from an anti-HBe positive asymptomatic patient before the development of an acute exacerbation following immunosuppressive treatment. After exacerbation, however, these deletion mutant clones disappeared or decreased in number and were replaced by clones with a stop codon mutant or TA mutant. These findings suggest that our 8-bp deletion mutant exert different effects on virus transcription and replication than those of the 8-bp deletion mutant described by Moriyama [1997].

On the other hand, in many DNA-dependent RNA polymerase II (pol II) dependent-promoter sequences, a "TATAA" sequence is located approximately 25 to 30 bp upstream of the initiation site. Interactions between pol II and other transcription factors with a "TATAA" sequence have been extensively studied, among which TATA-binding protein (TFIID: transcription factor II D) is known to play an important role in the critical initiation of mRNA. Transcription of numerous genes that do not contain a "TATAA" sequence is thought to be controlled by TATAA-less promoter. Although CP is TATAA-less promoter, the 3.5 kb mRNA of HBV has been identified to be transcribed by pol II of the host cell [Ganem and Varmus, 1987]. Assuming that some deletions are present in CP, possible changes in *cis*-acting element would occur and consequently, this might modify the transcription and replication activities of the virus.

In the present study, the in vitro transcriptional activity of naturally occurring mutant HBV with 8-bp deletion in CP was analyzed and then the underlying mechanisms of altered transcription activity was examined.

MATERIALS AND METHODS

Cells

The human hepatoblastoma cell line HepG2 was maintained in DMEM (Nissui, Tokyo, Japan) containing 10% fetal calf serum (FCS) (Filtron, Brooklyn, Australia) at 37°C under 5% CO_2 in air.

Construction of Recombinant Plasmids

Because HBV variants occurring naturally might possess various mutations throughout the entire HBV genome, recombinant plasmids in which the native function of the deletion in CP could be examined were constructed first. Plasmid pHB4-Del with an 8 bp-deletion (from nt. 1768 to nt. 1775) was generated by polymerase chain reaction (PCR)-based site-directed mutagenesis of pHB4, that originated from pHB125 containing HBV DNA of subtype *adr* at *BamHI* site of pBR322 [Fujiyama et al., 1983]. Two first-step PCRs were carried out with the forward primer HBV-Bm 5′-TCA TGG CGA CCA CAC CCG TC-3′, mutagenized reverse primer rHBV-Del 5′-CAG CCT CCA GAC CTT TAA CCT AAT C-3′, reverse primer rHBV-Bm 5′-TGA TGC CGG CCA CGA TGA GT-3′ and mutagenized forward primer HBV-Del 5′-GAT TAG GTT AAA GGT CTG GAG GCT GTA GGC AT-3′. The second PCR was carried out with primer HBV-Bm and rHBV-Bm, using the first-step PCR products. All PCRs were conducted with LA Taq kit (Takara Shuzo, Kyoto, Japan).

For the transfection experiments designed to examine the expression of HBsAg, HBeAg and progeny virus production, two recombinant plasmids were constructed with a head-to-tail dimer DNA of HBV of wild type and 8 bp-deletion mutant, respectively, by using pHB4 and pHB4-Del. The recombinant plasmids were constructed by the introduction of each *BamHI* digested full-length HBV fragment to the *BamHI* site of pUC19 and named pUC wt(2) and pUC Del(2), respectively.

The recombinant CAT plasmid, named pCAT pr/wt, was also constructed by inserting partial HBV genome containing the CP region (nt. 1604-1815), that was obtained from wild-type HBV *adr* subtype derived from pHB125, to the upstream of CAT gene of the pCAT basic vector (Promega, Madison, WI) lacking eukaryotic promoter and enhancer sequence. Similarly, in pCAT pr/Del, the CP region from the HBV derived from pHB4-Del was inserted into the pCAT basic vector.

Transfection

Three transfection experiments were carried out: 1) transfection of the recombinant plasmids with a head-to-tail dimer DNA to examine the expression of HBsAg, HBeAg and the production of progeny virus including HBV-specific RNA and DNA synthesis; 2) transfection of recombinant CAT plasmids (pCAT pr/wt and pCAT pr/Del) to examine CP activity; and 3) co-transfection of pCAT pr/wt and recombinant plasmid [pUC wt(2) and pUC Del(2)] to examine the possible change in *trans*-acting function of X-protein by deletion.

In all experiments, HepG2 cells grown in 60-mm^2 dishes were used. In the first type of experiment, 10 μg recombinant plasmids with a head-to-tail dimer DNA and 1 μg pXGD2 plasmid with a human growth hormone (hGH) gene (Nippon Gene, Tokyo, Japan) were

transfected using polycation in lipids (Lipofectine, Gibco BRL, Life Technologies, Inc., Rockville, MD). Briefly, plasmids were mixed with 10 μl Lipofectine solution in 1 ml of serum-free medium and the transfection mixture was overlaid on the cells. After incubation at 37°C for 8 hr, 3 ml of DMEM containing 5% FCS was added, and the dishes were further incubated for 16 hr. The culture medium was replaced with 5 ml of fresh DMEM containing 5% FCS after washing three times with phosphate-buffered saline (PBS). The culture medium was examined for the production of HBsAg, HBeAg and Southern blotting and the transfected cells were subjected to Northern blot analysis. In the second type of experiment to examine CP, 10 μg recombinant CAT plasmids with partial HBV genome containing the CP region were transfected. In the third type of experiment to examine *trans*-acting function of X-protein, 5 μg pCAT pr/wt and 5 μg recombinant plasmids with a head-to-tail dimer HBV genome DNA were transfected. The latter two types of experiments were carried out as described by Nishizono et al. [1995].

Detection of HBsAg, HBeAg and hGH

At 6 days after transfection, the production of HBsAg, HBeAg and hGH was determined by the Immunis HBsAg and HBeAg/Ab EIA kit (Institute of Immunology, Tokyo, Japan) and by hGH ELISA kit (Boehringer Mannheim, Mannheim, Germany), respectively, according to the protocol provided by the manufacturer.

Southern and Northern Blot Analysis

To detect progeny viral DNA, 4 ml of the culture medium harvested at 6 days after transfection were overlaid on 7.5 ml of 20% sucrose-PBS and centrifuged at 1.5×10^5 *g* with SW40 Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) for 16 hr. The resulting pellets were suspended in 1 ml of 10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA, 0.2 mg/ml proteinase K (Boehringer Mannheim) and 0.5% (w/v) SDS and further incubated at 37°C for 16 hr. DNA was extracted with phenol-chloroform and precipitated with ethanol, subjected to electrophoresis in 1% agarose gel, and blotted onto a nylon filter membrane (Hybond N+, Amersham Pharmacia Biotech, Uppsala, Sweden). Hybridization was carried out with a random-primed HBV whole genome probe labeled with [α-^{32}P] dCTP (Amersham Pharmacia Biotech).

Total RNA was extracted from transfected cells by the guanidinium thiocyanate phenol-chloroform method (ISOGEN, Nippon Gene, Tokyo, Japan). In the next step, 20 μg of RNA were subjected to electrophoresis in 1% formamide agarose gel and blotted onto a nylon filter membrane (Hybond N+, Amersham Pharmacia Biotech). The probe for Northern blot analysis was similar to that used for Southern blot analysis. The radioactivity of specific bands in Southern and Northern blot analyses was counted with the BAS 2000 image analyzer (Fuji Film, Tokyo, Japan).

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AdMLP :5′-AAG GGG GCC TAT AAA AGG GGG TGG-3′
       3′-TTC CCC CCG ATA TTT TCC CCC ACC-5′

NC    :5′-GAT TAT GGG CAT GCC GTA TTT-3′
       3′-CTA ATA CCC GTA CGG CAT AAA-5′
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Fig. 1. Nucleotide sequences of double stranded oligonucleotides used for probe and competitors in gel shift assay. The sequence of AdMLP was derived from adenovirus 2 major late promoter gene.

CAT Assay

At 3 days after transfection, CAT assay was carried out as described by Gormann et al. [1982] using 100 μg protein per reaction and incubation at 37°C for 1 hr. Ethylacetate-extracted samples (10 μg) were spotted on silica gel thin-layer plates. [^{14}C] Chloramphenicol and its acetylated forms, chloramphenicol 1-acetate and chloramphenicol 3-acetate were detected by autoradiography and the relative CAT activity was calculated using the BAS 2000 image analyzer.

RNase Protection Assay

The target region of the riboprobe used in the RNase protection assay for the precore and pregenome transcripts, consisted of CP and 3.5 kb mRNA initiation site, spanning 149 nucleotide length from nt. 1712 to nt. 1860. A DNA fragment was amplified by PCR using sense primer *SalI*-BCP (nt. 1713–nt. 1731, 5′-TGT CGA CTG TTT GTT TAA GGA CT-3′) and antisense primer rMγ (nt. 1841–nt. 1860, 5′-GTG GGA CAT GAA CAT GAC AT-3′). It was then inserted into pGEM-T vector (Promega) and subcloned. Riboprobe was synthesized by T7 RNA polymerase with MAXIscript T7 Kit (Ambion, Austin, TX) according to the instructions provided by the manufacturer. After running the synthesized riboprobe to 5% denatured polyacrylamide gel containing 6 M urea, the band representing the specific transcript was excised from the gel and purified in a solution containing 0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS.

In the next step, 100 μg of total RNA with 10^4 cpm riboprobe were subjected with RPAII kit (Ambion) according to the protocol provided by the manufacturer. RNA-RNA hybridization was carried out at 55°C for 16 hr. For RNase digestion, 10 units RNase T1 (Ambion) and 0.25 units RNase A (Ambion) were used to digest the RNA-RNA hybrids in 200 μl of digestion buffer (Solution Bx; described in the protocol provided by the manufacturer) at 37°C for 60 min. After ethanol precipitation, the protected RNA fragment was then analyzed in 8% denatured polyacrylamide gel containing 6 M urea. The radioactivity of specific bands was counted with a BAS 2000 image analyzer.

Gel Shift Assay

Gel shift assay was carried out as described previously [Hiraga et al., 1994] using HepG2 nuclear extract [Nishizono et al., 1991]. To evaluate the effect of mutations located in the CP region on binding of protein factors, two double-stranded (ds) partial genome of

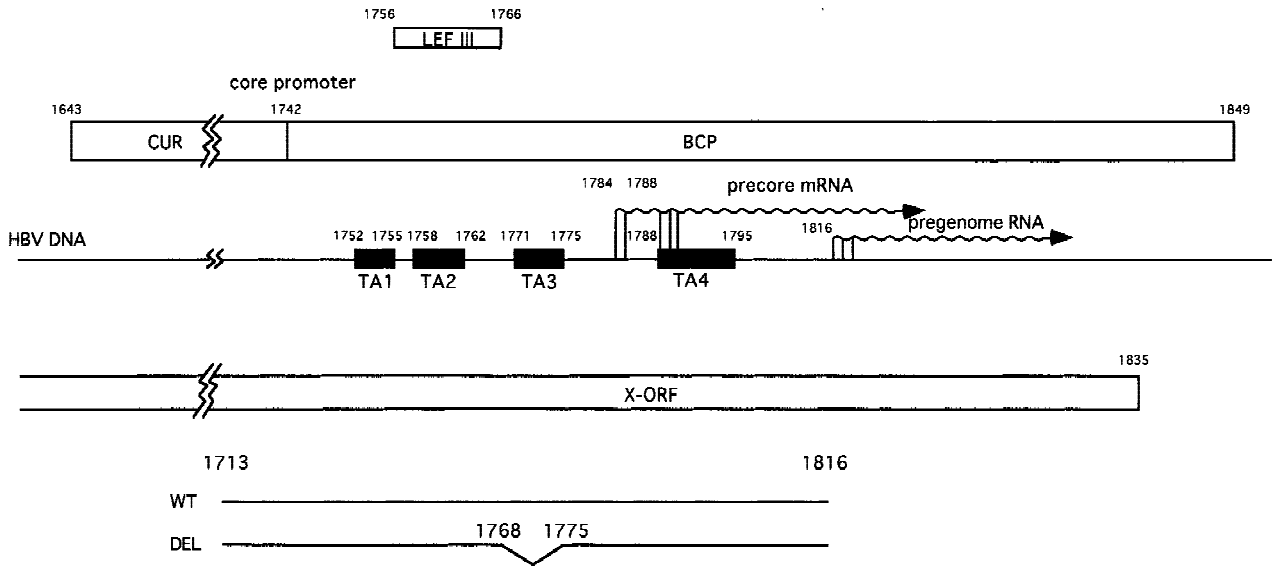

Fig. 2. Schematic diagram of the wild-type HBV CP region and map of the initiation site of precore and pregenome RNAs. Four TA-rich regions of the CP of HBV are shown as solid boxes. Numbers indicate nucleotide position on the HBV genome. Liver enriched factor (LEF) binding site, basic core promoter (BCP) region, core upstream region (CUR) and X ORF are shown as open boxes.

HBV *adr*4 (probe WT and competitor DEL) and two ds-oligonucleotides (competitor AdMLP and NC) (Figs. 1 and 2) were constructed. Probe WT is a 104bp ds-DNA corresponding to the partial genome of the HBV *adr*4 nt. 1713 to nt. 1816 and contains four TA rich regions. Competitor DEL is a 96bp ds-DNA containing 8-bp deletion from nt. 1768 to nt. 1775 of the probe WT. Competitor AdMLP is a 24 mer ds-oligonucleotide containing TFIID binding sequence (TATAA, underline in Fig.1) of adenovirus major late promoter region [Hirai et al., 1986]. Competitor NC is a non-specific 21 mer ds-oligonucleotide. The probe WT was labeled with [α-^{32}P] ddATP (Amersham Pharmacia Biotech) by 3′ end labeling kit (Amersham Pharmacia Biotech). The reaction mixture contained 0.5 μg HepG2 nuclear extract, 20 mM Tris-HCl pH 8.0, 80 mM KCl, 10 mM $MgCl_2$, 2 mM DTT and 10% glycerol. The mixture was preincubated on ice with or without the competitors for 30 min and was given labeled probe and incubated at 30°C for an additional 120 min. It was then subjected to electrophoresis in 5% non-denatured polyacrylamide gel, and the dried gel was autoradiographed using X-ray film.

RESULTS

Reduction of Viral Antigen Production and Replication of 8-bp Deletion Mutant

The capability of antigen and progeny virus production of recombinant HBV plasmids, pUC wt(2) and pUC Del(2), by transfection were determined. In order to confirm the transfection efficiency of the experimental conditions, pXGD2 plasmid containing hGH gene was co-transfected as an internal control and the amount of hGH in the culture supernatant was determined by ELISA. The amount of hGH was stable in all transfection experiments (Table 1). As shown in Table 1, production of HBsAg and HBeAg in pUC Del(2)-transfected culture media was less than that of wild-type, pUC wt(2) and production of both antigens equally reduced in pUC Del(2)-transfectant. In contrast, those in pUC Del(2)-transfectant were higher than those in mock (pUC 19)-transfected culture media, suggesting that the activity of antigen production of the deletion mutant remains at low level in natural infection with HBV variant having 8-bp deletion.

Southern blot analysis using pUC Del(2)-transfectant demonstrated that the ability of the mutant to produce progeny virus was very low (Fig. 3A). In addition, Northern blot analysis showed that the levels of pUC Del(2)-transcripts, such as precore mRNA, pregenome RNA and preS/S mRNA, were reduced markedly compared with that of pUC wt(2)-transcripts (Fig. 3B). The amount of 3.5 kb mRNA in pUC Del(2) was approximately 18% of that in pUC wt(2), that probably explained the low production of HBeAg (Table I). These findings suggest that the 8-bp deletion in the CP region is responsible for the reduced expression of HBsAg, HBeAg and reduced progeny virus production. These results add support to the previous study, in which it was demonstrated that 8-bp deletion mutant was a dominant clone during the asymptomatic anti-HBe positive phase, but not during the exacerbation phase.

Transcription Activity of 8-bp Deletion Mutant

The transcription activities of BCP were examined by using recombinant CAT plasmids with or without 8-bp deletion. The relative CAT activity of pCAT pr/Del transfectant harvested at 3 days after transfection was 44.8% of pCAT pr/wt transfectant (Fig. 4A). In the co-transfection experiments, pCAT pr/wt was transactivated by the authentic X protein produced by pUC wt(2). The CAT activity of pCAT pr/wt, however, did

TABLE I. Production of HBsAg and HBeAg in Culture Media of HepG2 Cells Transfected With a Wild-Type or a Deletion HBV Genome*

	hGH[a]	HBsAg[b]	HBeAg[b]
pUC19	0.510 ± 0.020	0.046 ± 0.001	0.048 ± 0.002
pUC wt (2)	0.525 ± 0.026	3.430 ± 0.085	3.710 ± 0.052
pUC Del (2)	0.516 ± 0.023	0.279 ± 0.019	0.464 ± 0.148

*Value represent the mean ± SD of optical density (O.D.) at 414 nm (a) and 490 nm (b) in ELISA. Transfection was performed in three independent experiments.

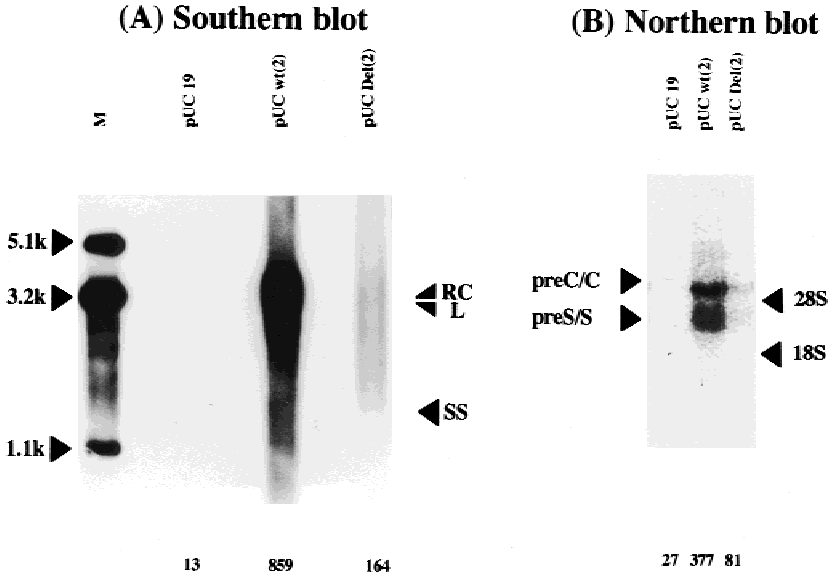

Fig. 3. (**A**) Southern blot hybridization of HBV DNA extracted from viral particles recovered from the culture medium of HepG2 cells 6 days after transfection of pUC wt(2) and pUC Del(2). Numbers at the bottom indicate the radioactivity counts of HBV-specific band by subtraction of background count. The migration position of relaxed circular (RC), linear (L) and single stranded (SS) DNA are indicated. (**B**) Northern blot hybridization of HBV RNAs extracted from the HepG2 cells 6 days after transfection as described in Southern blot analysis. The positions of the 3.5 kb precore mRNA and pregenome RNA (preC/C), and 2.1 and 2.4 kb mRNA (preS/S) are indicated (arrow head). The 18S and 28S ribosomal RNA markers are indicated (arrow head). The total radioactivities of HBV-specific bands counted by BAS 2000 are shown at the bottom.

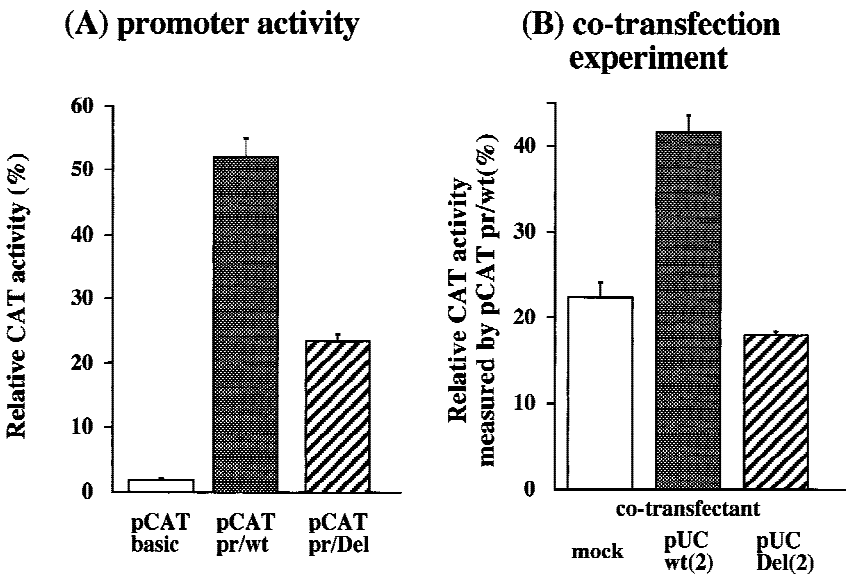

Fig. 4. (**A**) Relative CAT activity of HepG2 cells transfected with pCAT basic, pCAT pr/wt and pCAT pr/Del for analyzing promoter activity. (**B**) Relative CAT activity co-transfected with pCAT pr/wt and mock, pUC wt(2) or pUC Del(2) for analyzing X protein transactivating activity.

not change by co-transfection with pUC Del(2) that was designed to produce mutant X protein (Fig. 4B).

Because BCP controls the transcription of precore mRNA and pregenome RNA, CAT assay does not reflect individual transcription activity. Therefore, the RNase protection assay was undertaken to determine the intensity of each transcript. As shown in Figure 5 precore mRNA and pregenome RNA were apparently reduced in the deletion mutant compared with the wild-type. Interestingly, a slightly shorter transcript from the authentic pregenome RNA start site was detected in the RNAs obtained from pUC Del(2)-transfectant (Fig. 5A). Differences in band intensity between the short transcript of pUC Del(2) observed in the protection assay (Fig. 5A) and preC/C transcripts of pUC Del(2) (Fig. 3B) reflect the use of five times as much RNA in the latter experiments in order to detect the presence of a rare transcript. These results suggest

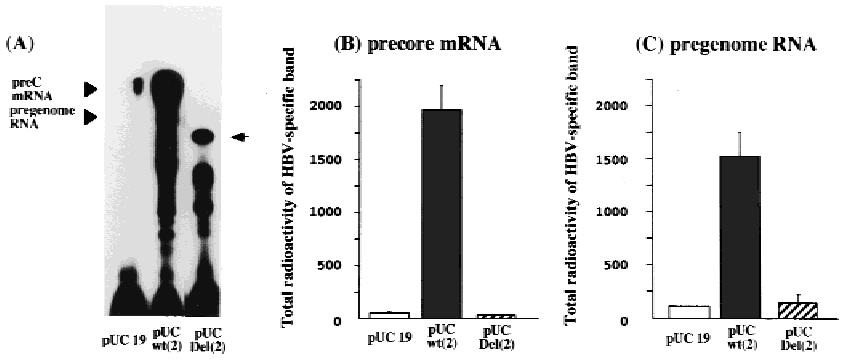

Fig. 5. Ribonuclease protection assay of 3.5 kb mRNA extracted from HepG2 cells 6 days after transfection with pUC Del(2). The position of precore mRNA and pregenome RNA are indicated (**A**). In this experiment, 100 μg of total RNA, corresponding to 5× as much as in Fig. 3B, were used. In (**B**) and (**C**), the radioactivities of the precore mRNA and pregenome RNA signals in authentic positions were counted by BAS 2000 image analyzer from triplicate transfection experiments, respectively. The level of statistical significance was determined by the unpaired test. $P < 0.001$ between pUC Del(2) and pUC wt(2) in (B), $P < 0.005$ between pUC Del(2) and pUC wt(2) in (C). Arrow indicates a short RNA transcript from an authentic pregenome transcription start site.

that 8-bp deletion in CP is probably the responsible factor not only for *cis*-acting reduction of precore mRNA and pregenome RNA transcription but also for the reduction of transcription via the loss of *trans*-acting activity of truncated X protein. Truncated X protein, lacking 20 amino acids from C-terminal end no longer exhibited transactivating activity on viral replication [Takada and Koike, 1994].

Effect of 8-bp Deletion on the Binding of Nuclear Proteins

Chen et al. [1995] demonstrated that TATAA-less promoter sequence of HBV contains four TA-rich sequences (TA1 through TA4) and directs transcription of precore and pregenome messages. Our 8-bp deletion mutant completely lost the TA3 region, that might be a possible cause of reduced promoter function.

In the next step, gel shift assay was carried out to determine the responsible mutation regulating 3.5 kb mRNA transcription. Four bound complexes (I, II, III and IV in Fig.6) were demonstrated when probe WT was used. These complexes were competed out by increasing amounts of cold probe (Fig. 6, lanes 3, 4, 5), however, the bands did not disappear after addition of large amount (100-fold excess) of competitor NC, suggesting that bands are specific for CP of HBV genome. The first, second and third bound complex formation was inhibited by increasing the amount of competitor DEL but the IVth was not inhibited (Fig. 6, lane 6–8). On the other hand, only the IVth was inhibited by increasing the amount of competitor AdMLP containing TATAA (TFIID binding sequence) (Fig. 6, lane 9–11). These results suggest that competitor DEL lacks a binding activity or has low affinity to TFIID.

DISCUSSION

HBV DNA 8-bp deletion 1763 to 1770 and 20-bp deletion 1753 to 1772 [Okamoto et al., 1994] can potentiate virus replication and show higher levels of transcription of their pregenomic RNA than the wild type despite the presence of low levels of expression of HBsAg, HBcAg and HBeAg [Moriyama, 1997]. The 8-bp deletion mutant (nt. 1768 to nt. 1775), however, showed a reduced activity to transcribe both precore mRNA and pregenome RNA and to produce progeny virus. HBsAg and HBeAg expression were also markedly inhibited. The reason for the discrepancy in the ability of pregenome RNA transcription and virus replication which was described by Okamoto et al. is not known at present. The following changes, however, might explain these differences. Examination of 30 nucleotides sequences from nt. 1750 to nt. 1779 in BCP of wild-type, shows the following sequence: 5′-GAT TAG GTT **AAA GGT CTT T**GT ACT AGG AGG-3′, in which the bold type indicates liver enriched factor (LEF) [Buckwold et al., 1996] binding sequence and the underlined indicate the first, second and third TA-rich regions in the order proposed by Chen et al. [1995]. The mutant with 8-bp deletion from nt. 1768 to nt. 1775 completely lacks TA3. Consequently, TA-rich sequence in BCP is shorter than that of wild-type and its *cis*-acting function might probably be altered compared with the authentic promoter activity. In fact, alteration of the *cis*-acting element caused suppression of BCP function as observed with reduced precore mRNA and pregenome RNA transcriptions (Figs. 4A and 5). In contrast, 8-bp deletion described by Moriyama [1997] lacks the whole sequence between TA2 and TA3 (nt. 1763 to nt. 1770), thereby TA2 and TA3 are connected together and generate a novel long TA-rich sequence as follows: 5′-GAT TAG GT**T AAA** TAC TAG GAG G-3′, in which the underlined represents the novel TA-rich region. On the other hand, the LEF region becomes shorter in the mutant. Buckwold et al. [1996] have demonstrated that mutation in the LEF region gives rise to reduced transcription of precore mRNA. Thus, these newly generated TA-rich sequence and consequent mutation in LEF might lead to reduced transcription of precore mRNA without affecting or with enhanced transcription of pregenome RNA.

Northern blot analysis showed that transcription of preS/S mRNA was also reduced in the mutant (Fig. 3B). This effect may be expected as a result of altered function of enhancer II, which overlaps the deleted basic core promoter region. This might be an expected event since changes in the enhancer function (Enh II) overlapping located in basic core promoter region by 8-bp deletion might lead to reduced transcription of preS/S mRNA.

The region encompassing TA1 to TA3 is crucial for precore mRNA transcription. Transcription of precore mRNA in a mutant with two point mutations in TA3

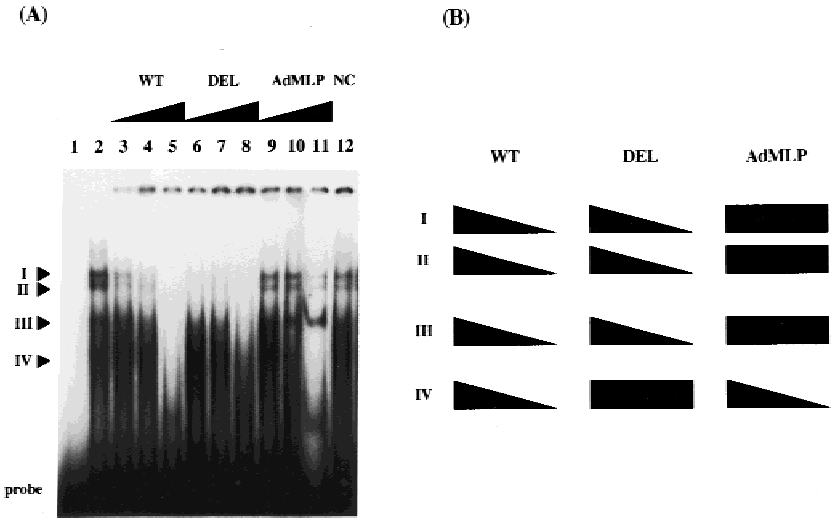

Fig. 6. (**A**) Gel shift assay with ^{32}P-labeled WT probe with three types competitors and HepG2 nuclear extract. Four specific bound complexes (I, II, III and IV) are indicated. Complex formation was monitored by competition with 1-, 10- and 100-fold excess of unlabeled WT, DEL, AdMLP, and NC competitors. **Lane 1**: without nuclear extract; **lane 2**: no competitor; **lanes 3–5**: with WT competitor (1-, 10- and 100-fold excess of probe, respectively); **lanes 6–8**: with DEL competitor (1-, 10- and 100-fold excess of probe, respectively); **lanes 9–11**: with AdMLP competitor (1-, 10- and 100-fold excess of probe, respectively); **lane 12** with NC competitor (100-fold excess of probe, respectively). (**B**) Schematic diagram of gel shift assay.

has been shown to be suppressed, whereas transcription of pregenome RNA was increased in the mutant. On the other hand, transcription of precore mRNA in a mutant with two point mutations in TA4 was not affected but that of pregenome RNA was inhibited [Chen et al., 1995]. Based on above observations, mutations in TA3 and TA4 cause an inhibitory effect on the transcription of precore mRNA and pregenome RNA, respectively. The 8-bp deletion mutant lacks completely the sequence of TA3 but TA4 sequence remains intact. The transcription of pregenome RNA, however, was apparently inhibited. Gel shift assay in the present study revealed that the IVth band observed with probe WT was not competed with the DEL sequence, suggesting that the mutant has altered binding activity between DNA dependent RNA polymerase II and CP. In other words, the nucleotide change in CP due to the lack of TA3 in the mutant generates new mutation and might result in failure to form the IVth bound complex. It is unclear from the present study which regulatory function was affected by the nucleotide change in CP by complete lack of TA3. It might be possible, however, that initiation of pregenome RNA transcription decreased in the mutant by nucleotide change in the upstream region of TA4. A shorter RNA detected in the RNase protection assay of the RNAs obtained from pUC Del(2)-transfectant (Fig. 3A) might be an outcome induced by such abnormalities in transcription regulation.

Another possibility might be an involvement of X protein. In most cases of deletion mutants, X-ORF overlapping with CP leads to frame-shifts and the termination of translation. Because X protein possesses the *trans*-activating function, viral transcription might be affected and the levels of transcripts might be altered during replication in deletion mutants. In the co-transfection experiments, the wild-type promoter activity for transcription of precore mRNA and pregenome RNA was transactivated only when co-transfected with authentic X protein (Fig. 4B). This might be ascribed to the truncated X protein generated in the 8-bp deletion mutant. According to our preliminary experiments using pUC wt(2) co-transfected with pUC Del(2), however, the levels of HBe and HBs Ag, progeny virus production and mRNAs transcription were not different from those of mock (pUC 19) co-transfection experiment (data not shown). Furthermore, the level of HBe Ag of pUC Del(2) was not enhanced even by the co-transfection with the recombinant plasmid expressing X protein (data not shown). Altered core promoter activity due to 8-bp deletion might be more responsible for reduced transcription than X protein *trans*-acting function.

It is possible that the type of 8-bp deletion mutant described in the present study might affect the propagation of concomitant wild-type HBV and causes subclinical and persistent infection. A silent HBV infection, in which serological markers are not detected, might develop potentially into an acute exacerbation and fulminant hepatitis.

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